# Mutagenicity of Trichloroethylene and Its Metabolites: Implications for the Risk Assessment of Trichloroethylene

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This article addresses the evidence that trichloroethylene (TCE) or its metabolites might mediate tumor formation via a mutagenic mode of action. We review and draw conclusions from the published mutagenicity and genotoxicity information for TCE and its metabolites, chloral hydrate (CH), dichloroacetic acid (DCA), trichloroacetic acid (TCA), trichloroethanol, *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC), and *S*-(1,2-dichlorovinyl) glutathione (DCVG). The new U.S. Environmental Protection Agency proposed Cancer Risk Assessment Guidelines provide for an assessment of the key events involved in the development of specific tumors. Consistent with this thinking, we provide a new and general strategy for interpreting genotoxicity data that goes beyond a simple determination that the chemical is or is not genotoxic. For TCE, we conclude that the weight of the evidence argues that chemically induced mutation is unlikely to be a key event in the induction of human tumors that might be caused by TCE itself (as the parent compound) and its metabolites, CH, DCA, and TCA. This conclusion derives primarily from the fact that these chemicals require very high doses to be genotoxic. There is not enough information to draw any conclusions for trichloroethanol and the two trichloroethylene conjugates, DCVC and DCVG. There is some evidence that DCVC is a more potent mutagen than CH, DCA, or TCA. Unfortunately, definitive conclusions as to whether TCE will induce tumors in humans via a mutagenic mode of action cannot be drawn from the available information. More research, including the development and use of new techniques, is required before it is possible to make a definitive assessment as to whether chemically induced mutation is a key event in any human tumors resulting from exposure to TCE.
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Trichloroethylene (TCE), an important industrial chemical and widespread environmental contaminant, is currently undergoing a thorough evaluation and risk assessment by The National Center for Environmental Assessment (NCEA) of the U.S. Environmental Protection Agency (U.S. EPA). As a part of this process, NCEA commissioned a series of reviews addressing issues of TCE toxicity and the possibility that TCE might present a human health hazard. TCE is known to be a rodent carcinogen and a neurotoxicant (*1*). Evaluating the rodent bioassay data for TCE presents an interesting challenge because it induces quantitatively different cancer responses in mice and rats. There is also some human epidemiological information concerning whether TCE is a human carcinogen (*2*). The possible modes of action by which TCE and its metabolites cause biological damage have been extensively studied. Possible modes include: somatic cell mutation, the proliferation of peroxisome enzymes, cytotoxicity and reparative hyperplasia, oxidative stress, alterations in calcium ion homeostasis, mitochondrial dysfunction, alterations in gene expression, cell proliferation, $\alpha_{2u}$-globin, and alterations in cell repair. Other articles in this issue (*3,4*) explore the possible nongenotoxic modes of action. Our article addresses the interpretation of the literature concerning whether TCE or its metabolites might mediate tumor formation via a mutagenic mode of action. We review and draw conclusions from the mutagenicity and genotoxicity information published for TCE and its metabolites, chloral hydrate (CH), dichloroacetic acid (DCA), trichloroacetic acid (TCA), trichloroethanol, *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC), and *S*-(1,2-dichlorovinyl) glutathione (DCVG).

## Proposed New U.S. EPA Cancer Risk Assessment Guidelines

The 1996 proposed revisions to the U.S. EPA Cancer Risk Assessment Guidelines emphasize the importance of mechanistic information in conducting a holistic evaluation of the available information for each chemical undergoing risk assessment. Generally the available data for cancer induction, following exposure to a chemical, have been obtained at doses far exceeding those to which a human would ever be exposed. In the past, it was generally assumed that chemicals that cause cancer at high exposure levels would also cause cancer at environmentally relevant exposures and that a linearized multistage model was appropriate for conducting a dose–response assessment using the available tumor data. This practice was based upon the general understanding that tumors are induced by chemical genotoxicants and on the assumption that the process occurred via linear kinetics.

Under the new guidelines, it is recognized that there are many factors involved in the induction of tumors and that cancer can result via a variety of mechanisms that may not operate by linear kinetics. The new process of cancer risk assessment involves the identification of key events (key modes of action) for specific tumor induction. Because of the importance of mutation in the etiology of tumors, mutagenicity and genotoxicity data are used to assess whether mutation is the, or a, key event by which a chemical under consideration might induce tumors.

## Drawing Proper Conclusions from Multiple Genotoxicity Assays

Because of the large number of genotoxicity assays, it can be difficult to draw a simple answer to the question: Is the chemical mutagenic? Unfortunately, most current analyses of these large databases are often no more than a simple assessment as to whether there is a clear pattern of positive data, a clear pattern of negative data, or a combination of positive and negative data. When there is a combination of positive and negative data, often the individual making the evaluation makes a decision on the basis of the relative number of positives and negatives. On the other hand, the assessor may call a mixture of positive and negative responses inconclusive. Either approach is a mistake and does not recognize the fact that different assays detect

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different types of mutational or genotoxic damage and that not all published data are of sufficient quality to be used to make definitive conclusions.

We feel that, with proper interpretation, much more information can be drawn from genotoxicity data. In this article, we outline our strategy for evaluating genotoxicity data and then apply this approach to the assessment of whether mutation is likely to be a key event in the induction of TCE-induced tumors.

We need to emphasize that our approach to this evaluation represents a new strategy, and while we feel it provides additional evidence in defining whether mutation induction is the (or a) key event in tumor induction, it does not provide the definitive answer. The critical question in cancer dose–response assessment involves understanding the totality of the mode(s) of action for a particular chemical, in the target tissue, and at environmentally relevant exposure levels. Genotoxicity tests, just like rodent cancer bioassays, are often conducted at very high exposure levels. The advantage of the genotoxicity tests, however, is that many of them are sensitive enough to detect damage at exposure levels far lower than can be used in the rodent bioassays. As described below, we utilize this feature in drawing conclusions concerning the possibility that a chemical will be genotoxic at environmental exposure levels.

## Definition of Genotoxic and Mutagenic

Before proceeding further, terminology must be defined. The term genotoxic is widely used and abused. It is used to define chemicals that cause DNA damage and/or mutations. It is also used, more narrowly, to define just those chemicals that form DNA adducts and/or are positive in the *Salmonella* bacterial gene mutation assay. For this review, the term mutagenic will be used to refer to the demonstration that a chemical can induce heritable mutations (damage that can pass to daughter somatic cells). Genotoxic will be used in a broader sense and will include mutational end points, cytogenetic analysis, and the evaluation of primary DNA damage.

## Assays That Detect Genotoxicity but Not Mutagenicity

The ability of a chemical to induce primary DNA damage is assessed in assays that detect DNA adducts, the analysis of DNA strand breakage, and unscheduled DNA synthesis (UDS). These assays provide evidence that the chemical can reach the DNA and either bind to it, cause the chromosomes or DNA strand(s) to break, or cause the DNA to undergo an unscheduled period of synthesis. Primary DNA damage assays do not necessarily reflect the adverse impact that a chemical might cause to the cell. While the formation of DNA adducts or strand breakage is often the first step in mutation induction, this damage can also be repaired. This results in no damage to the cell, or the cell's repair systems may be saturated, causing the cell to invoke a signal transduction pathway resulting in programmed cell death. Therefore, it is important to remember that DNA adducts or strand breakage assays do not demonstrate that an agent is mutagenic.

Microscopic analysis of metaphase chromosomes (cytogenetic analysis) provides information as to whether a chemical can cause a change in chromosome number or break chromosomes, resulting in a variety of aberrations. While cytogenetic analysis identifies the ability of the chemical to cause structural damage to chromosomes, such damage may not be compatible with cell survival. Primary DNA damage information and cytogenetic analysis may provide key pieces of information, but it is much more useful to know whether a chemical has the ability to induce DNA or chromosomal damage that can be passed to daughter cells. This type of damage, a mutation, is involved in the process of tumor formation.

## Evaluation of Mutagenicity

The induction of tumors is a multistep process and, at its basis, a genetic, mutational disease. Mutations become involved in the etiology of cancer by chemical (or physical agent) induction or by increased cellular growth that provides for an increased spontaneous mutation frequency and/or clonal expansion of preexisting mutations. Because these different processes have different kinetics, understanding the mode(s) of action of specific carcinogens provides insight into the shape of the dose–response curve.

There is a broad array of mutational damage, from simple point mutation (base pair changes, insertions, or deletions), to chromosomal rearrangement (translocations, mitotic recombination, and gene conversion) and deletion, to loss or gain of chromosomes (aneuploidy), to changes in the whole chromosome complement (polyploidy). Over the course of the past several years, new molecular techniques coupled with cytogenetic analysis have revealed that all of the various types of mutational damage can be involved in the etiology of tumors.

When conducting a thorough hazard characterization for a chemical, it is important to determine if that chemical can induce any of the various types of possible mutations. The different mutation hazard identification assays have different abilities to detect these changes. The widely used *Salmonella* assay detects only a small subset of the possible types of mutational damage (point mutations, a few base pair deletions and insertions). Therefore, chemicals that do not induce point mutations, yet cause chromosomal mutations or aneuploidy, would be expected to be negative in the *Salmonella* assay and positive in an assay that detects chromosomal aberrations or chromosomal mutations. Because it is necessary to evaluate chemicals for their ability to cause point mutations and chromosomal mutations, the *Salmonella* assay alone is insufficient for mutation assessment.

There are a very large number of mutation assays, the majority of which evaluate the induction of a mutation at a particular gene. These assays use a variety of cells in culture (primarily bacterial, yeast, fungi, or mammalian cells) or whole organisms (primarily rodents). When reviewing, analyzing, and interpreting genotoxicity data, it is important to recognize the differences among the various types of tests.

## The U.S. EPA-Recommended Genotoxicity Battery

The U.S. EPA requirements and recommendations for genotoxicity assay selection are detailed in Dearfield et al. (*5*). This recommended battery includes the *Salmonella* mutation assay, the *in vitro* mouse lymphoma (thymidine kinase gene) mutation assay, and an *in vivo* analysis of chromosomal damage. It should be noted that the mouse lymphoma assay, although it uses a single gene as the selectable marker, detects both gene mutations and chromosomal mutations (*6*). The *in vivo* component of this battery is, in fact, not a mutation assay, but rather a cytogenetic assay. While not ideal, this selection was made because of the dearth of appropriate *in vivo* gene mutation assays.

## Integration of the Available Genotoxicity Information

Ideally, a risk assessment to determine a chemical's mutagenic potential should be based on a complete data set. Unfortunately, unless the chemical evaluation is done in a systematic manner, specifically for risk assessment, not all the critical information is generally available. In such situations, it is important to remember that lack of information does not equate to lack of activity. That is, if there are little or no available data, it is incorrect to say that there is no evidence that the chemical is mutagenic. Rather, it is correct to say that there is no information as to the possible mutagenicity of the chemical.

When there is information from multiple assays and end points, how should those data be weighed and integrated? The answer depends upon the question being asked. When, as for TCE, the goal is to provide

insight into the cancer dose response, one of the key pieces of information is whether the TCE-induced tumors result from TCE-induced mutations. In the absence of this information (due to the lack of adequate techniques), data that define the genotoxicity or mutagenicity of TCE (or its metabolites) in the target organ should receive the most weight. Of next importance is the definition of the *in vivo* (nontarget tissue) mutagenicity of the chemicals. It should be emphasized, however, that there are currently no techniques that can completely define the nonmutagenicity of a chemical *in vivo* in either target or nontarget tissue. The techniques that can be used *in vivo* to detect mutational damage do not cover the full range of possible mutations important in the etiology of cancer. At a minimum one needs to determine if the chemical can induce either point mutations or chromosomal damage. Presently this must be done using both gene mutation and cytogenetic assays. While there are some gene mutation assays that can be applied to target tissue, cytogenetic analysis is generally not possible. Only in rare situations (as with DCA—see below) is there both *in vivo* gene mutation (in this case in target tissue) and cytogenetic data (in this case in bone marrow). Therefore, while in general one might like to assign less weight to *in vitro* information, such information is still critical in the overall assessment. Thus, chemicals that are not mutagenic *in vivo*, yet are clearly demonstrated to be mutagenic *in vitro*, should not automatically be ruled as nonmutagenic.

Generally, mutagenicity data should receive more weight than primary DNA interaction data. One exception is when the primary DNA interaction data are obtained *in vivo* and in the target organ. Positive data obtained using the target tissue provide powerful evidence that the chemical or its metabolite reached the DNA in the target organ.

Of course, these various assays provide information as to whether a chemical has the potential to be genotoxic or mutagenic. They do not provide definitive information as to whether the chemical induces tumors by a mutagenic mechanism. In fact, with the currently available techniques, it is not possible to conclusively determine whether a tumor is induced via a chemically induced mutational mechanism. At best, one can consider the available information and evaluate the likely mechanisms.

## Use of Genotoxicity/ Mutagenicity Data in Dose–Response Assessment

Genotoxicity data provide information concerning a chemical's potential to cause mutational damage and, in the context of cancer risk assessment, the potential to mediate that tumor formation through a mutational mechanism. Historically, in the risk assessment arena, mutagenic carcinogens were assumed to induce tumors with linear dose–response kinetics and thus not to have thresholds or shallow initial dose–response curves resembling thresholds. This is not, in fact, a good assumption. There are many theoretical reasons why mutagenic carcinogens might have nonlinear dose–response curves. The low-dose linear default assumption is based on the assumption that mutations are induced by one-hit kinetics and that even one molecule of the chemical has the potential to be mutagenic. Observed during early studies with radiation, one-hit kinetics occur when one chemical interaction with the DNA results in one mutation. It has long been known that chromosomal mutations, such as interstitial deletions involving multiple base pairs and translocations, require two hits (two chemical interactions) with the DNA or chromosome and thus have nonlinear dose–response curves. For chemicals acting primarily by inducing chromosomal mutations with two-hit kinetics, a linear extrapolation from data obtained at high dose levels will greatly overestimate the risk at low dose levels.

Furthermore, the shape of the dose–response curve for mutagenic carcinogens is not driven solely by the kinetics by which a chemical and/or its metabolite(s) induces mutations relevant to tumor formation. In all cases the chemical must, at a minimum, reach the target organ(s). Most chemicals are metabolized, and this is often a prerequisite to mutagenicity or carcinogenicity. The kinetics by which a chemical moves through the organism and is metabolized and distributed in the tissues is likely to strongly affect the shape of the tumor or mutation dose–response curve.

Often rodent cancer bioassays require relatively high doses of chemicals over long time periods to induce tumors. Because the biological consequences of mechanisms active at these high doses may be different from those of mechanisms active at lower doses, it is possible that tumors may be induced only at these high doses and that low doses may result in no tumor formation. Alternatively, the shape of the tumor dose–response curve may be very different at high and low doses. Like tumors, mutations also can be induced at high doses by mechanisms that are not operational at lower doses. A properly conducted genotoxicity evaluation battery of tests will provide some insight into the ability of a chemical to induce mutations across a full dose range. This issue is particularly important in predicting the likely shape of tumor dose–response curves at environmentally relevant exposure levels. Chemicals that induce mutations only at high doses (or are nonmutagenic) are highly unlikely to have linear tumor dose–response curves across all exposure levels.

## Review of Data for TCE and TCE Metabolites

As stated above, genotoxicity data provide evidence concerning the potential for a chemical to mediate cancer through a mutational mechanism. Unfortunately, it is impossible at present to determine whether tumors are actually mediated by chemically induced mutations. The DNA sequence analysis of mutations present in tumor tissue can, in some situations, provide insight into this issue, and there is some DNA sequence information available for TCE and its metabolites DCA and TCA.

This article reviews the genotoxicity information for TCE and for its metabolites. Each chemical is addressed and summarized separately. In each case, available data are evaluated to determine the likelihood that the chemical is mutagenic, and also whether the chemical is likely to be mutagenic at environmentally relevant doses. This assessment is made by considering the magnitude of the dose required to show any observable genotoxic effect. As will be seen below, the doses required for TCE and its metabolites to induce any genotoxicity are generally orders of magnitude higher than one would expect to occur in a target organ. The differences between the rodent and human metabolism of TCE and the differences in the concentrations of the various metabolites in the target tissues are important factors in the interpretation of the probable mode of action. This information is well detailed by other authors in this series of papers (*3,4,7*).

Following the individual chemical evaluations, the information is integrated to provide an assessment of all the available genotoxicity information and how it relates to the potential TCE human cancer health risk.

### Trichloroethylene

Trichloroethylene has been extensively studied for potential genotoxicity and mutagenicity. Because there are extensive reviews summarizing this body of literature (*8,9*), only a summary of the data and their interpretation will be presented. Taken together, evidence from a number of different analyses and a number of different laboratories using a fairly complete array of end points indicates that TCE may have the potential to be both genotoxic and mutagenic. However, the evidence that TCE is mutagenic is not convincing and is somewhat confounded by the fact that TCE is often stabilized with a very low concentration of epichlorohydrin or 1,2-epoxybutane, both known to be potent mutagens (*10*). Available evidence indicates that if TCE is genotoxic/mutagenic, it must be metabolized to induce genetic damage.

The most relevant observations from the genotoxicity and mutagenicity information are summarized below. In assays that evaluate the ability of the chemical to interact or directly damage DNA, there is evidence that metabolites of TCE can bind to DNA (*11,12*). Of particular interest with regard to the observed differences between the mouse and rat following TCE exposure, Miller and Guengerich (*12*) found that when treated with TCE, isolated mouse hepatocytes showed severalfold higher DNA adduct levels than isolated rat hepatocytes. In some studies TCE appeared capable of inducing point mutations (*8*). However, in a series of carefully controlled studies evaluating TCE (without the mutagenic stabilizers), McGregor and co-workers (*10*) found TCE incapable of inducing point mutations in *Salmonella.* Therefore, it is unlikely that TCE induces point mutations.

There is some evidence that TCE may be a weak inducer of other types of mutational damage, including mitotic recombination and aneuploidy (*13–15*). Technical deficiencies in the conduct of the *in vitro* mammalian cell mouse lymphoma assay (no colony sizing) limit the usefulness of the *in vitro* mammalian data (*16*). *In vitro* gross chromosome aberration analysis using Chinese hamster ovary cells found TCE to be negative both with and without exogenous activation (*17*). *In vivo,* however, there is some evidence that TCE or its metabolites can bind to DNA in hepatic and kidney cells, and that it can also induce single-strand DNA breaks in both hepatic and kidney cells (*11,18*). Some species differences were observed when single-strand DNA breakage was analyzed in hepatic cells by alkaline unwinding. Mice required less TCE exposure to cause single-strand DNA breaks than rats (*18,19*). There is some evidence that TCE can induce bone marrow cell micronuclei in rats but not mice, but unfortunately the study was compromised by high negative controls in one (rat) data set, which prevented the confirmation of the rat response (*20*). TCE was weakly active in the mouse spot test, which determines the ability of chemicals to induce gene mutations or recombination *in loci* governing the color of the animal's coat (*21*). Several investigations have involved humans occupationally exposed to TCE (*22,23*). Sister chromatid exchange (SCE), aneuploidy, and chromosome aberrations in peripheral lymphocytes, as well as sperm morphology have been analyzed (*24–27*). No clear positive effects have been observed in these human studies, but all of the studies have one or more technical deficiencies. Given the number of studies conducted, however, if TCE were a potent inducer of genetic damage in peripheral lymphocytes, it is likely that damage would have been observed.

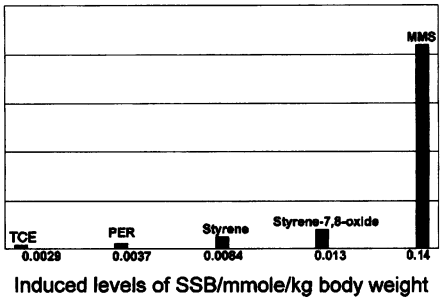


**Figure 1.** A comparison of the capacity for induction of single-strand breakage (SSB) in liver DNA of mice treated with different agents. The values were determined 1 hr after injection and were normalized to 1 mmol/kg/ body weight. The induced level of SSB is expressed for TCE, PER, styrene, styrene-7,8-oxide, and MMS. All data are from Walles (*19*).

An evaluation of the potential genotoxic potency of TCE shows that the dose of TCE required to give any positive responses is generally very high. In the various assays in which TCE has been evaluated, the concentration required to see mutagenicity is very high relative to the concentration required for other chemicals to show genotoxicity. That is, the assays used are capable of detecting responses at doses orders of magnitude lower than required to see TCE activity. The studies of Walles (*19*) help put the potency of TCE for inducing DNA single-strand breaks in the livers of male mice into perspective. Using intraperitoneal (i.p.) injection and the DNA unwinding technique, researchers compared the capability of methylmethanesulfonate (MMS), styrene-7,8-oxide, styrene, perchloroethylene, and TCE for inducing single-strand DNA breaks (*19*). From this study (Figure 1) it is clear that even at the very high doses used, 4–10 mmol of TCE/kg body weight, TCE induced only very low levels of DNA breakage. The absence of DNA breakage at lower exposure levels argues that very high exposure levels of TCE must be attained before it is able, either directly or through its metabolites, to break DNA. It is unlikely that these exposure levels would ever occur in humans exposed to TCE.

In summary, in those genotoxicity assays in which positive responses were seen following TCE exposure, the activity generally was weak, even at very high doses. It also appears that TCE itself has little, if any, mutagenic potential; any mutagenic activity resulting from TCE exposure likely results from one or more of its metabolites.

## Chloral Hydrate

Chloral hydrate has been extensively studied as a potentially genotoxic agent. It has been evaluated in the recommended genotoxicity screening battery and several other assays including genetic alterations in rodent germ cells. CH was positive in bacterial mutation tests, indicating that it may be capable of inducing point mutations (*28–30*). It was positive in the mouse lymphoma assay for mutations at the Tk locus (*31*). The mutants were primarily small colony Tk mutants, indicating that most CH-induced mutants resulted from chromosomal mutations rather than point mutations. In other *in vitro* assays, CH induced micronuclei in Chinese hamster embryonic fibroblasts (*32*), Chinese hamster pulmonary cell lines Luc2 and Don.Wq.3H (*33*), and human peripheral blood lymphocytes (*34*). Aneuploidy induction (assessed as kinetochore positive micronuclei) was observed in Chinese hamster Luc2 cells (*35*) and human peripheral blood lymphocytes (*36*). Chromosome aberrations were found in Chinese hamster embryonic diploid cells (*37*).

Because there is a mixture of positive and negative *in vivo* data, with no reason to weigh some studies more than others, it is not clear whether CH is capable of inducing genetic damage *in vivo.* Russo and Levis (*38*) found CH to be capable of inducing aneuploidy in mouse spermatocytes. Two different research groups reported an increase in micronuclei in mouse spermatids when treatment involved exposure of spermatogonia stem cells (*39,40,*) and Russo et al. (*41*) found CH to induce micronuclei in mouse bone marrow erythrocytes. Other studies, however, found CH to be negative in *in vivo* experiments (*42,43*), and a study with mouse oocytes also found CH to be negative (*44*).

Although CH can induce a variety of mutational events, it does so with a very low potency. Figure 2 shows the relative potency in the *Salmonella* assay for CH and other metabolites of TCE. Its relative potency for inducing micronuclei is shown in Figures 3 and 4. The data in Figures 3 and 4 were generated as a part of the European Communities collaborative study to evaluate a number of assays for their ability to detect aneugens. Figure 3 shows the comparative data from Ferguson et al. (*36*), using human peripheral blood lymphocytes; Figure 4 shows the data from Lynch and Parry (*35*), using low-passage Chinese hamster Luc2 cells. In both cases, the number of micronuclei induced by CH was relatively low and the concentration required to induce that effect quite high. In the *in vitro* mouse lymphoma assay, capable of detecting almost the complete spectrum of mutational events, CH was one of the least potent mutagens evaluated (Figure 5).

The comparative potency data (Figures 2–5) are provided to emphasize the evidence that the mutagenic activity of CH is likely a high-dose effect. As shown clearly in these figures, the *Salmonella* assay, the micronucleus assays, and the mouse lymphoma assay can

respond to reflect the genetic activity of chemicals at doses much lower than were active for CH. Stated another way, if CH were genotoxic at lower levels, these assays would have detected that activity. In all of these *in vitro* assays, the concentration of CH required to see a positive response was over 500 µg/mL, a concentration likely several orders of magnitude greater than would ever be reached *in vivo* in a target tissue. Therefore, we conclude that it is unlikely that any TCE-induced tumors would be mediated through mutations induced by CH.

## Dichloroacetic Acid

The standard genotoxicity screening battery found DCA to be mutagenic. In *Salmonella typhimurium* strain TA 100, S9 enhanced the activity of DCA significantly when the vaporization (Tedlar bag) technique was used (*46*). Evidence that DCA can induce point mutations is strengthened by the DeMarini et al. (*46*) observation that the molecular spectrum of mutants in DCA-treated cultures was different from that of untreated cultures.

DCA was mutagenic in the *in vitro* mouse lymphoma assay (*31*), inducing primarily small colony mutants (indicating chromosomal mutations). These researchers also found DCA to be clastogenic *in vitro* to mouse lymphoma cells. Fuscoe et al. (*52*) utilized a drinking water route of administration with DCA *in vivo*, and determined that DCA induced a very small increase in the number of bone marrow micronuclei when animals were exposed to concentrations similar to those used in the rodent bioassay.

DCA can cause DNA strand breaks in mouse and rat liver cells following *in vivo* administration by gavage (*18*). Interestingly, the DNA strand break dose–response curves differ between mouse and rat. Fuscoe et al. (*52*), using the single-cell gel (or comet) assay, reported crosslinking in blood leukocytes in mice exposed to 3.5 g/L DCA for 28 days. As discussed, primary DNA damage assays, although useful in determining the ability of a chemical to reach the target tissue and interact with the genetic material, do not prove that the chemical can cause mutational damage.

More informative with regard to the possibility that DCA can cause mutations in liver cells is the study using the Lac I locus in the Big Blue mouse (*53*). These investigators used a drinking water route and the same doses of DCA that cause tumors in rodents. Mice treated with 3.5 g/L DCA for 60 weeks had a 2.3-fold increase in mutant frequency over the concurrent controls. Mutational spectral analysis of these mutants (in which approximately 1,400 base pairs were analyzed for possible mutation) revealed a different spectrum in the mutants in DCA-treated animals than was seen in the untreated animals, indicating that the mutations were likely induced by the DCA treatment. Also

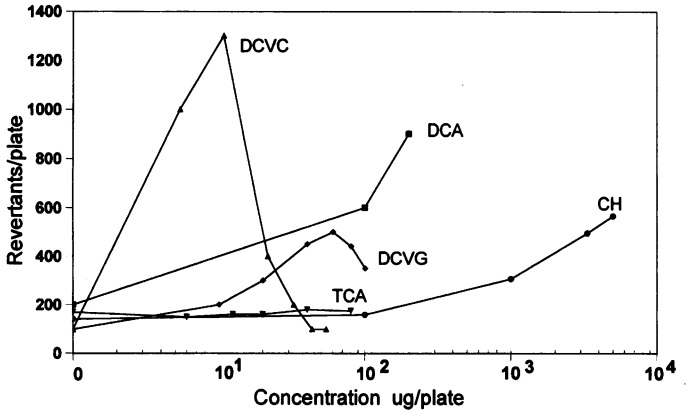


**Figure 2.** Mutagenicity of TCE metabolites in *Salmonella* strains TA100 and TA2638. DCVC ▲ and DCVG ◆ were evaluated using strain TA2638; data from Vamvakas et al. (*45*). DCA ■ and TCA ▼ were evaluated using a vaporization technique in *Salmonella* TA100; data from DeMarini et al. (*46*). CH ● was evaluated in strain TA100; data from Haworth et al. (*29*).

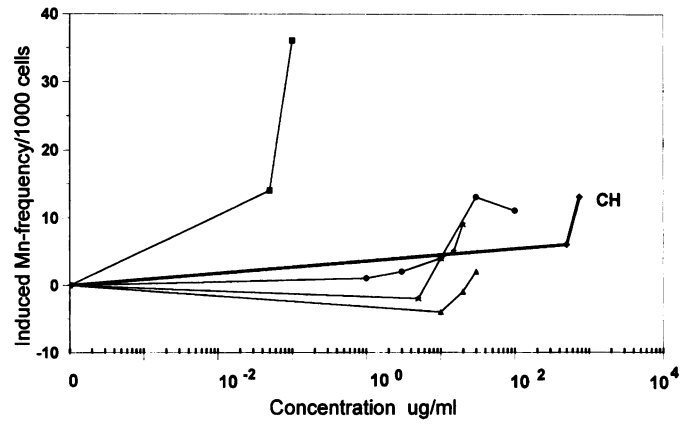


**Figure 3.** Effects of various compounds on the frequency of micronuclei in cytokinesis-blocked human peripheral blood lymphocytes; colchicine ■; chloral hydrate ◆; diazepam ●; econidazole ▲; hydroxoquinone ★. Data are from Ferguson et al. (*36*).

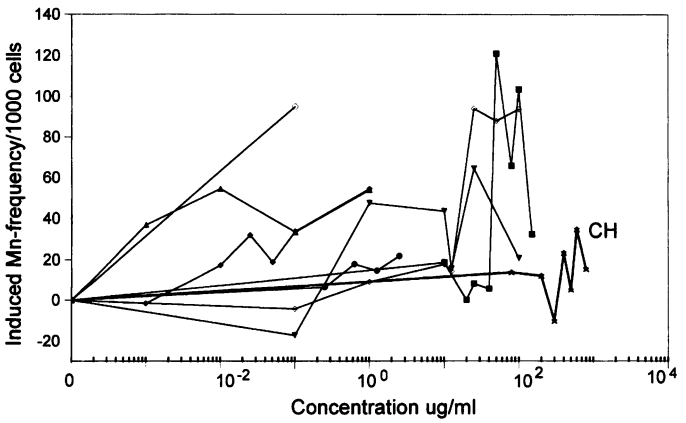


**Figure 4.** Comparative micronucleus induction in low-passage Chinese hamster Luc2 cells. Chloral hydrate ★; colchicine ◆; vinblastine ▲; thiabendazole ■; diazepam ◇; pyrimethamine ▼; cadmium chloride ●; and mitomycin C ○. All data are from Lynch and Parry (*35*).

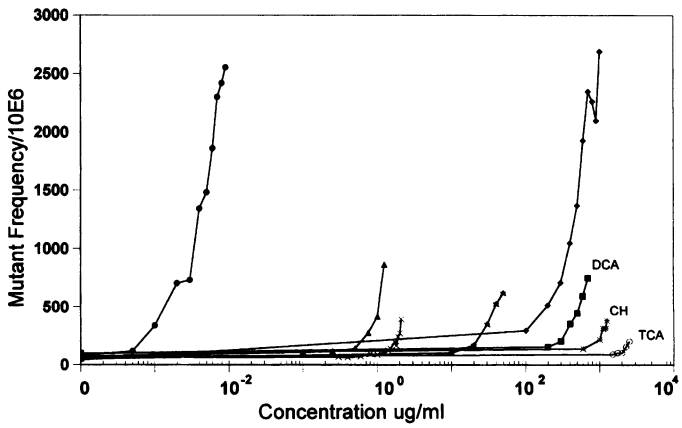


**Figure 5.** A comparison of Tk mutant frequencies after treatment of L5178Y/Tk$^{+/-}$-3.7.2C cells with *m*-amsacrine ●, DeMarini et al. (*50*); 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5*H*]-furanone (MX) ▲, Harrington-Brock et al. (*47*); Arsenic ×, Moore et al. (*48*); 2-acetylaminofluorene ★, Harrington-Brock (*51*); ethylmethansulfonate ◆, Moore et al. (*49*); DCA ■, CH ✳, and TCA ○, Harrington-Brock et al. (*31*).

of significance, the types of mutations observed were similar to those previously seen in codon 61 of the H-*ras* gene of liver tumors from mice treated with DCA (see further discussion below).

At the high dose of DCA, a large portion of the liver may actually be tumor tissue. Every attempt, however, was made to use only normal tissue rather than tumor tissue for the analysis (*54*). These precautions were taken because tumors result from clonal expansion, and the presence of tumor tissue in the sample evaluated would give a falsely high mutant frequency if a Lac I mutation occurred in the rapidly expanding tumor clone. The investigators were able to eliminate the possibility that clonal expansion played a significant role because the DNA sequence analysis demonstrated that almost all of the mutations were unique.

When all of this DCA information is integrated, it is clear that DCA is mutagenic but only very weakly mutagenic. This conclusion is based on comparing the potency of DCA and other chemicals in the *Salmonella* assay (Figure 2), the *in vitro* mouse lymphoma assay (Figure 5), and *in vivo* for micronuclei induction (Figure 6). In all three of these assays, DCA is one of the least potent mutagens that have been evaluated. Furthermore, in the Big Blue mouse system, the amount of DCA required to induce a very small mutagenic response is very high (Figure 7).

As can be clearly seen in Figures 6 and 7, the *in vivo* assays are capable of demonstrating substantial responses at doses much lower than were required for DCA. Therefore, if DCA were capable of inducing large amounts of genotoxic (or mutagenic) damage, this could have been detected in either of the *in vivo* assays. Therefore, while one cannot eliminate the possibility that DCA might induce tumors via a mutagenic mode of action, we feel that the weight of the evidence argues that any TCE-induced tumors would not be mediated by DCA-induced mutation.

## Trichloroacetic Acid

Trichloroacetic acid is the least mutagenic of the TCE metabolites discussed so far. It was negative in *Salmonella* (*46*) (Figure 2). TCA was weakly positive in the mouse lymphoma assay. However it was one of the very least potent mutagens (Figure 5) identified in the assay (*31*) and was substantially less mutagenic than either DCA or CH. It is unclear whether TCA can induce chromosomal damage *in vivo*, because some studies have seen positive responses and others negative. Nelson and Bull (*18*) and Nelson et al. (*59*) found TCA to break hepatic DNA strands, and Bhunya and Jena (*60*) and Birner et al. (*61*) found TCA to be genotoxic in mouse and chick test systems. In contrast, Mackay et al. (*62*) used a neutralized form of TCA and found that TCA does not induce chromosome damage in human lymphocytes *in vitro* or in the bone marrow micronucleus test. These results with the neutralized TCA support the observation that TCA is nonclastogenic (*63*) and the negative results obtained by other researchers (*64,65*). It is therefore unlikely that TCA would contribute to tumor formation through a mutational mechanism.

## Trichloroethanol

Trichloroethanol was negative in the *Salmonella* assay (*46*) but has not been evaluated in the other recommended screening assays. As noted already, the *Salmonella* assay detects only a small subset of the possible types of mutations. One cannot conclude that a chemical is nonmutagenic until it has been evaluated in assays that detect chromosomal mutations. The potential of trichloroethanol as a mutagen, therefore, remains unknown.

## Trichloroethylene Conjugates

DCVC and DCVG are capable of inducing point mutations, as evidenced by their mutagenicity in bacteria (*45,66*). DCVC is the most potent of the TCE metabolites as a *Salmonella* mutagen (Figure 2), while DCVG appears to be substantially less potent and is similar in potency to DCA. DCVC is a metabolite of DCVG (*7*), and based on the *Salmonella* data, might be argued to be the most active of the metabolites.

It should be noted that the data shown in Figure 2 represent results in two different *Salmonella* tester strains. This creates somewhat of a compromise in the interpretation of the data. DCVC and DCVG were evaluated in TA2638, a strain with increased metabolic capacity. The other chemicals, DCA, TCA, and CH, were not evaluated in TA2638 but rather were evaluated in TA100, a strain lacking the increased metabolic capacity. Therefore, there is some possibility that DCA, TCA, and CH could, in fact, be more potent in a bacterial point mutation system than would be indicated by Figure 2.

Unfortunately, there is no genotoxicity information for DCVC and DCVG in the other recommended screening tests. There is, however, some indication that DCVC can induce primary DNA damage in mammalian cells *in vitro* and *in vivo* (*67,68*). In a Syrian hamster embryo fibroblast system, DCVC gave a comparatively weak UDS response and no induced micronucleus formation (*69*). In addition, Vamvakas and Koster (*70*)

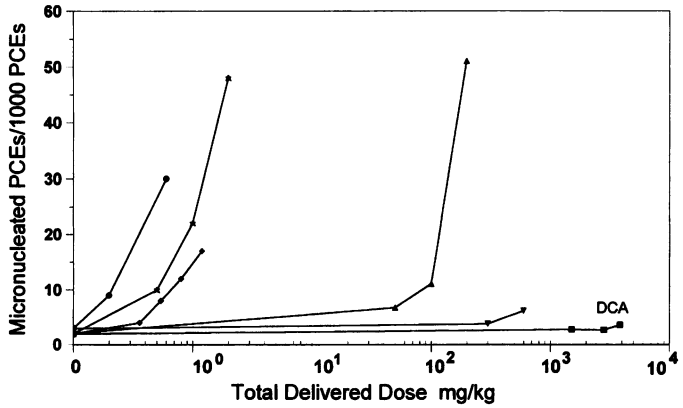


**Figure 6.** A comparison of the induction of micronucleated polychromatic erythrocytes in peripheral blood of mice. Male B6C3F$_1$ mice were treated with 1.0, 2.0, and 3.5 g/L of DCA ■ in drinking water (9 days *ad libitum*). Data shown are calculated total doses from Fuscoe et al. (*52*). Male B6C3F$_1$ male mice received 3 i.p. injections of benzidine ▼ and 7,12-dimethylbenzanthracene ▲. Data shown are calculated total doses from Tice et al. (*55*). Male Swiss-Webster mice received a single i.p. injection of either triethylenemelamine ●, mitomycin C ★, or colchicine ◆; data are from MacGregor et al. (*56*).

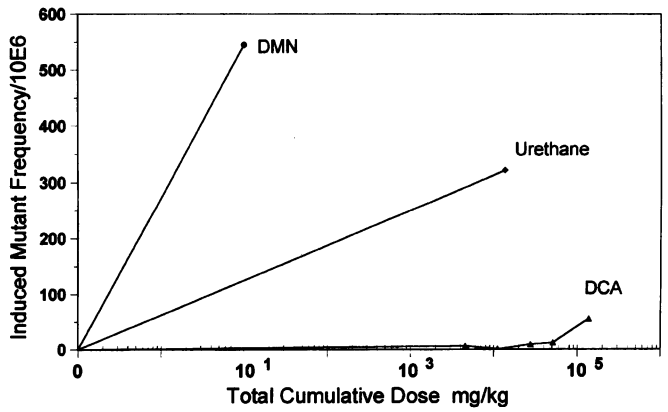


**Figure 7.** A comparison of mutant frequencies induced in liver cells of Big Blue transgenic mice. Urethane ◆ was administered in the feed for 105 days; data from Shephard et al. (*57*). Dimethylnitrosamine (DMN) ● was administered i.p. for 5 days; data from Mirsalis et al. (*58*). DCA ▲ was administered in the drinking water at 1.0 g/L and 3.5 g/L for 4, 10, or 60 weeks; data shown are from Leavitt et al. (*53*) with total cumulative dose data provided by DeAngelo (*77*).

have shown that DCVC can induce the expression of two protooncogenes, c-*fos* and c-*myc*. It is unknown whether this alteration in gene expression might be involved in tumor induction.

In summary, DCVC (a metabolite of DCVG) is the most potent bacterial mutagen of the TCE metabolites. Because there are no data for mammalian cells from *in vitro* or *in vivo* experiments, the genotoxic potency of DCVC and DCVG is unknown.

## Analysis of Mutational Spectra in Tumors

As stated in the introduction, the key question is whether TCE-induced tumors are mediated through an induced mutational mechanism. That is, is chemically induced mutation a key event in the induction of TCE tumors, or do tumors arise primarily through some other mode of action such as increased cell proliferation, which serves to increase the spontaneous mutation frequency? The kinetics of these two modes of action would be very different, and thus this distinction is critical for dose–response assessment. Several research groups (*65,71,72*) investigating the etiology of mouse liver tumors in untreated and chemically treated animals have analyzed mutations in the *ras* oncogene of tumor tissue. All of these studies reported that approximately 50% of the tumors from either untreated or treated animals had a mutation in either K-*ras* or H-*ras*. For instance, in the Ferreira-Gonzalez et al. (*65*) study, 45–50% of the tumors from DCA-treated animals showed mutations in Exon 1, 2, or 3 of H-*ras* or K-*ras*. In the Anna et al. study (*71*), 59% of the tumors from DCA-treated animals showed mutations in H-*ras* codon 61. Untreated animals show very similar percentages of H-*ras* mutations in their tumors, with these two studies reporting 58 and 54%, respectively. It is therefore important to note that K-*ras* or H-*ras* mutation is obviously not required to induce tumors and that other events are important, and may be rate limiting, either in addition to or instead of *ras* gene mutation.

Both Anna et al. (*71*) and Ferreira-Gonzalez et al. (*65*) compare the mutations seen at codon 61 of H-*ras* in treated and untreated animals (Table 1). Because the number of tumors analyzed in these studies is very small, information from Maronpot et al. (*72,73*) is presented in Table 2. In the untreated animals in these four studies, 52–62% of the mutations are to AAA; 26–36% are to CGA; and 10–12% are to CTA. For animals treated with TCE, the number of mutants that are AAA is much lower and the number of CTA mutants is much higher than in untreated animals. Animals treated with DCA show a decrease in AAA mutants and an increase in CGA and CTA mutants. The TCA-treated animals show a slight increase in the AAA mutants and no CTA mutants. However, in all cases, particularly for TCA, the number of mutations analyzed is relatively small. In fact, for TCA only five mutants were analyzed, making it difficult to draw any valid conclusions. Taken together these data suggest that the percentage of the specific point mutations is different in the tumors from DCA- and TCE-treated animals. While it could be argued that this is consistent with a chemically induced mutational mechanism, the actual amount of mode-of-action information provided by this approach is quite minimal.

All four of the above studies were conducted using male mice. Other researchers using female $B6C3F_1$ mice found very few codon 61 H-*ras* mutations in tumors from DCA-treated animals (*74*). Therefore, it is clear that codon 61 H-*ras* mutation is certainly not required for liver tumor formation.

As stated above, the approach used in these studies is not likely to yield definitive mode-of-action information. The male $B6C3F_1$ hybrid used in these studies has a high occurrence (approximately 40%) (*72*) of spontaneous hepatocellular neoplasms. This makes it impossible to distinguish spontaneously arising tumors from chemically induced tumors. Furthermore, the analysis of mutational spectra at any three base pair codon, where spontaneous mutation occurs, lacks the resolving power needed to determine whether the mutations seen are spontaneous or induced. Full utilization of the power of mutational spectral information requires the analysis of entire genes or substantial portions of genes. In addition, the most information is obtained by a two-pronged approach that includes both an analysis of mutations in oncogenes or tumor-suppressor genes in tumor tissue and the determination of the full array of possible mutations that can be induced by the chemical being evaluated. This evaluation of the full array of possible mutations requires the use of a target gene that can be mutated by the chemical and can display all the possible mutations that the chemical can induce. Concordance between mutations observed in cancer-relevant genes in tumor tissue and possible mutations that can be induced by the chemical provides evidence (but does not prove) that the chemical is causing tumors by a mutational mechanism. Such studies have not been done for TCE or any of its metabolites.

Of course, the key questions are whether TCE can induce tumors in humans and whether those tumors are induced through a mutational mode of action. A group of German researchers are trying to address this issue by evaluating the spectrum of mutational events seen in the von Hippel-Lindau (VHL) gene of renal cell carcinomas from people who have been exposed to high levels of TCE (*75,76*). Like the studies in rodents, the pattern of mutations found by these researchers appears to be different in tumor tissue taken from people exposed and not exposed to TCE. Unlike the rodent studies discussed above, these investigators are sequencing large numbers of base pairs and thus may be able to identify specific mutational patterns in tumors from individuals exposed to TCE. However, as with all human studies, the subjects of these investigations likely were occupationally exposed to a variety of chemicals, which makes any definitive determination difficult.

Recently, this research group (*76*) has found what appears to be a specific mutational hot spot at nucleotide 454 in the VHL gene of renal cell carcinomas from individuals occupationally exposed to TCE. In this study the paraffin-embedded tumor tissue from 44 people with renal cell carcinoma and known exposure to TCE were evaluated. VHL mutations were found in 33 (75%) of the cases. Often there were multiple mutations and also loss of heterozygosity of the VHL gene. The nucleotide 454 mutation was seen in 13 (39%) of the cases. In addition, this same mutation was present in adjacent nonneoplastic kidney parenchyma in 4 of these patients. This implies that this mutation occurred rather early in the etiology of the tumors in these 4 patients. The TCE-exposed individuals were compared to 107 renal cell carcinoma patients who had no known TCE exposure. While VHL mutations were observed in the tumor tissue of people not known to be exposed to TCE, none of these mutations occurred at nucleotide 454. While this study does not prove that TCE induced

**Table 1.** Mutation analysis of codon 61 (CAA) in H-*ras* of liver tumors from $B6C3F_1$ male mice treated with TCE or its metabolites.

| | Total mutations, no. | AAA | | CGA | | CTA | |
|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % |
| Control | 77 | 48 | 62 | 20 | 26 | 8 | 10 |
| DCA | 62 | 15 | 24 | 25 | 40 | 22 | 35 |
| TCA | 5 | 4 | 80 | 1 | 20 | 0 | 0 |
| TCE | 42 | 12 | 29 | 10 | 24 | 17 | 40 |

Combined data of Anna et al. (*71*) and Ferreira-Gonzalez et al. (*65*).

**Table 2.** Mutation analysis of codon 61 (CAA) in H-*ras* of liver tumors from untreated $B6C3F_1$ mice.

| | Total mutations, no. | AAA | | CGA | | CTA | |
|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % |
| Female | 33 | 17 | 52 | 12 | 36 | 4 | 12 |
| Male | 177 | 106 | 60 | 50 | 28 | 21 | 12 |

Data from Maronpot et al. (*72,73*).

the VHL mutations in these individual, these data are particularly intriguing and do suggest a potential role for TCE in the etiology of the VHL gene alterations.

## Summary Evaluation

From the available data, it is clear that TCE and its metabolites are not potent genotoxic agents. When compared with other genotoxic or mutagenic agents in a number of different test systems, both *in vitro* and *in vivo*, the results (Figures 1–7) consistently show that TCE and its metabolites (with the exception of DCVC) are weakly genotoxic and require high doses to induce a response. The concentrations required to attain positive responses in the various *in vitro* and, where information is available, *in vivo* assays are much higher (orders of magnitude higher) than one would expect to see, *in vivo*, in any possible target tissue. We interpret this body of evidence to suggest that most of the genotoxic activity of these chemicals (with the exception of DCVC) is likely due to mechanisms that occur only at relatively high concentrations—levels that would not be attained *in vivo*. Therefore, we feel it is unlikely that these chemicals would be mutagenic, at any target tissue, at exposure levels that are relevant to humans. When all of the information concerning the genotoxic potential for TCE and its metabolites is taken together, it appears unlikely that TCE could induce tumors through a mutagenic mode of action.

Unfortunately, however, we cannot eliminate the possibility that chemically induced mutation is a key event in the development of TCE-induced tumors. DCVC does cause point mutations, at least in bacteria. It is unfortunate that so little data are available for this chemical. It is not clear whether the relatively potent (compared to the other TCE metabolites) response seen *in vitro* in *Salmonella* would also be observed in an *in vivo* assay.

Also, as indicated earlier, the key questions are whether the human tumors associated with TCE exposure are in fact induced by TCE, and if so, whether chemically induced mutation is a key event in that tumor induction. While there is much research yet to be done, the studies analyzing the kidney tumors from humans with high exposure to TCE are proving to be very interesting. However, if TCE is capable of inducing mutations, it is surprising that the standard battery of mutation assays is unable to detect this mutagenicity. Further studies, including studies to elucidate all the possible types of mutations that may be induced by TCE (or TCE metabolites—particularly DCVC) should provide significant new information. Further investigation of trichloroethanol and the other relevant trichloroethylene conjugates is also required. These studies combined with the human investigations should provide substantial new information that may change the conclusions that we have drawn concerning whether chemically induced mutation is likely to be a key event in the etiology of TCE-induced tumors.

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